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(54) Title: ASPARAGINE HYDROXYLATION OF THE CAD DOMAIN OF A HIF PROTEIN



(57) Abstract: A target asparagine residue of HIF 1 alpha and 2 alpha is hydroxylated at high oxygen tension to render HIF as a weak transcription factor. An asparagine hydroxylase hydroxylation motif and binding motif is proposed. A method of screening for agonists or antagonists of an asparagine hydroxylase is also proposed and involves mixing peptides or proteins having the hydroxylation and or the binding motif with asparagine hydroxylase and a candidate agonist or antagonist. The extent of inhibition or enhancement of binding; level of asparagine hydroxylation or transactivation may be measured depending on the nature of the protein or peptide. Additionally altered proteins resistant to hydroxylation are described as are nucleic acids encoding such proteins.

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## ASPARAGINE HYDROXYLATION OF THE CAD DOMAIN OF A HIF PROTEIN

## FIELD OF THE INVENTION

- 5 This invention relates to an altered HIF protein having modified asparagine hydroxylase binding and hydroxylation motifs, proteins or peptides exhibiting such modified motifs, nucleic acids encoding such modified motifs and cells carrying such nucleic acids. Additionally the invention relates to a method of isolating agonists or antagonists of hydroxylation of the asparagine hydroxylation of HIF.

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## BACKGROUND OF THE INVENTION

- Normal human physiology is dependent upon a continual supply of oxygen. When oxygen becomes limiting, cells of affected tissues undergo a number of adaptive responses to aid survival. These include metabolic adjustments, for example, switching
- 15 energy production from oxidative phosphorylation to glycolysis, as well as genetic reprogramming events aimed at increasing oxygen supply to tissues. Key genes which are activated during hypoxia (low oxygen stress) include those which encode erythropoietin (EPO), a growth factor which increases the production of oxygen carrying red blood cells; Vascular Endothelial Growth Factor (VEGF), a protein which promotes
- 20 new blood vessel development; and a set of genes which produce enzymes involved in glycolysis as well as a number of other changes.

- A number of oxygen sensitive mediators have been identified. Two proteins which sense depleted oxygen levels and subsequently act as transcription factors (gene regulatory
- 25 factors) to induce the above mentioned genes are the Hypoxia Inducible Factors  $1\alpha$  and  $2\alpha$  (HIF- $1\alpha$  and HIF- $2\alpha$ ).

- HIF- $1\alpha$  and HIF- $2\alpha$  are two closely related transcription factors. The schematic of Figure 1 shows these proteins exhibit a similar organisation of functional domains. The
- 30 N-terminal bHLH/PAS domains are important for dimerisation and DNA binding; the Oxygen Dependent Degradation Domains (ODDs) mediate protein turnover; and the

There are several documents that describe some aspects of the behaviour of the CAD domain of HIF in normoxic conditions compared to hypoxic conditions however none of these has identified the hydroxylation of a target asparagine ( O'Rourke *et al.*, 1999, Ema *et al.*, 1999, Carerro *et al.*, 2000, Gu *et al.*, 2001, Jiang *et al.*, 1997). The term CAD refers to a functional transactivation domain at the C-terminus of the HIF-1 $\alpha$  and HIF-2 $\alpha$  proteins and different laboratories use the term to describe varying lengths of the C-terminus. We define the CAD as the hypoxia inducible C-terminal transactivation domain contained within the last 100 amino acids of HIF-1 $\alpha$  and HIF-2 $\alpha$ .

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#### SUMMARY OF THE INVENTION

This invention arises from the finding that a target asparagine residue of HIF 1 $\alpha$  and 2  $\alpha$  is hydroxylated at high oxygen tension, to thereby render HIF as a weak transcription factor. Additionally other amino acids of the HIF protein have been identified as being  
15 important in hydroxylation of the target asparagine.

The target asparagine that is subject of the hydroxylation occurs within a motif that is conserved as between HIF 1  $\alpha$  and 2  $\alpha$  as well as between mouse and human. An hydroxylation motif adjacent the hydroxylated asparagine is also highly conserved and  
20 seems likely to be important in the hydroxylation. O'Rourke *et al.*, (1999) also found that alteration of the amino acid triplet RLL at positions 774-776 in HIF-1 $\alpha$  are essential for oxygen modulation of the transactivation function of HIF-1 $\alpha$ . Similarly adjacent amino acids are highly conserved and it is proposed that these constitute a binding motif, and that alteration in these will interfere with the action of the asparagine hydroxylase. It is  
25 found that the RLL sequence is critical for the binding of the asparagine hydroxylase to the CAD domain in pull-down assays.

The invention might in a first broad aspect be said to reside in a DNA binding protein having an altered HIF CAD domain, whereby the alteration inhibits hydroxylation of the  
30 asparagine but maintains the capacity of the altered CAD domain to exert its

## BRIEF DESCRIPTION OF THE DRAWINGS

- Figure 1 Is an illustration of the overall structure of the HIF-1 $\alpha$  and HIF-2 $\alpha$  protein showing the location of the domains present on a linear amino acid map, and also showing amino acid homologies between the two HIF proteins for certain of the domains. bHLH refers to the basic Helix-Loop Helix; PAS refers to the Per-Arnt-Sim homology region; CAD, refers to C-Terminal transactivation domain and ODD refers to oxygen dependent degradation domain.
- Figure 2 Is an illustration of the oxygen dependent mechanism in place to regulate the turnover and CAD activity of the HIF-1 $\alpha$  and HIF-2 $\alpha$  proteins. bHLH refers to the basic Helix-Loop Helix; PAS refers to the Per-Arnt-Sim homology region; CAD, refers to C-Terminal transactivation domain; ODD refers to oxygen dependent degradation domain and VHL refers to the von-Hippel-Lindau factor.
- Figure 3. Expression vectors for either the Gal4 DNA Binding Domain or the indicated GalDBD HIF-1  $\alpha$  and HIF-2  $\alpha$  CAD chimeric proteins were cotransfected with a Gal4 response element containing luciferase reporter gene and an internal control renilla luciferase reporter gene. Transfected HEK293T (A) or PC12 (B) cells were left untreated, or subjected to hypoxia or 2,2'-Dipyridyl (DP) treatment for 16 h, before luciferase activities were measured by the dual luciferase assay (Promega). Data are the average of three transfections +/- standard deviation.
- Figure 4. A) Schematic showing domains of HIF-2 $\alpha$  and the construct containing the CAD region fused to a 6xHis tag / myc epitope which was used to

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sequence at  $m/z$  values of +16 Da higher than Asn851-containing fragments of the unmodified sequence. The boundary of the coincident and +16 Da fragment ions is indicated by the symbol N+ on the fragment ion spectrum of the modified sequence.

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Table 2. Theoretical and observed masses of the indicated ions derived from tandem MS sequencing of the hydroxylated (2106 column) and non-hydroxylated (2090 column) peptide YDCEVNVVPVPGSSTLLQGR [SED ID NO. 1]. A difference of 16 Da occurs when a hydroxyl moiety is present on the peptide.

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Figure 6. Mutation of asparagine confers strong, constitutive activity to the HIF-1 $\alpha$  and HIF-2 $\alpha$  CADs. A) Sequence comparisons show conservation of the critical Asn between HIF-1 $\alpha$  and HIF-2 $\alpha$  across species. B) Reporter gene assays showing constitutive activity of the mutants GalDBD/HIF-1 $\alpha$  727-826(N803A) and GalDBD/HIF-2 $\alpha$  774-874(N851A). HEK293T cells were transfected according to the protocol of Figure 3A and data represent the average of triplicate transfections  $\pm$  standard deviation. C & D) The wild type and mutant GalDBD/HIF chimeras used in A) are expressed at the same levels during transient transfection. Whole cell extracts of control or transfected cells were separated by SDS-PAGE and proteins detected by immunoblotting with antibodies directed against the C-terminus of HIF-1 $\alpha$  (C) or HIF2 $\alpha$  (D).

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Figure 7. Importance of selected amino acids within the HIF CAD asparagine hydroxylation motif. Recombinant HIF-1 $\alpha$  CAD, either wild type or point mutant where alanine has replaced the indicated amino acid, was incubated with purified, recombinant HNH in a reaction cocktail

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digested with SalI/XbaI and cloned into SalI/XbaI digested GalO. This is thus a vector encoding a chimeric protein. Unique restriction site NarI was introduced by silent mutation. C) represents the complete HIF-1 $\alpha$  cloned into pefbos-cs. The HIF-1 $\alpha$  has had an N803A amino acid substitution. D) represents a vector with HIF 2 $\alpha$  fragment 774 - 874. The was made by PCR amplifying DNA encoding 774-874 incorporating BamHI, myc and NdeI sites), digested PCR product with BamHI/NotI digested EF.HIF-TD 727-826.puro 6.

## 10 DETAILED DESCRIPTION OF THE INVENTION

By way of a shorthand notation the following three and one letter abbreviations for amino acid residues are used in the specification as defined in Table 1.

Where a specific amino acid residue is referred to by its position in the polypeptide of a protein, the amino acid abbreviation is used with the residue number given in superscript (i.e. Xaan)

TABLE 1

20 Amino Acid	Three-letter Abbreviation	One letter Abbreviation
Alanine	Ala	A
Arginine	Arg	R
25 Asparagine	Asn	N
Aspartic Acid	Asp	D
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic acid	Glu	E
30 Glycine	Gly	G

It will be understood that the invention is applicable to variants of the HIF proteins where variations occur outside of the CAD domain. Thus deletions of the protein, for example, deletion of all or part of ODD may be preferred in that this domain enhances protein turnover which turnover is mediated by a prolyl hydroxylase, and it may be desired to  
5 maintain function of the protein at hypoxia and normoxia. The entire ODD may be deleted to achieve this, thus providing a truncated HIF protein having dimerisation and DNA binding domains as well as CAD domain. Alternatively the proline subject of hydroxylation may simply be substituted so that ubiquitination cannot be achieved. Additionally other modification may be made to the HIF protein. Thus for example it is  
10 known that simple amino acid substitutions do not materially affect the function of the protein. In particular, conservative amino acid substitutions might be made.

One or more amino acid residues within the sequence can be substituted by another amino acid of a similar polarity, which acts as a functional equivalent, resulting in a silent  
15 alteration. Substitutes for an amino acid within the sequence may be selected from other members of the class to which the amino acid belongs. For example, the nonpolar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan and methionine. Amino acids containing aromatic ring structures are phenylalanine, tryptophan, and tyrosine. The polar neutral amino acids  
20 include glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine. The positively charged (basic) amino acids include arginine, lysine and histidine. The negatively charged (acidic) amino acids include aspartic acid and glutamic acid.

Particularly conservative amino acid substitutions are:

- 25 (a) Lys for Arg or vice versa such that a positive charge may be maintained;
- (b) Glu for Asp or vice versa such that a negative charge may be maintained;
- (c) Ser for Thr or vice versa such that a free OH can be maintained;
- (d) Gln for Asn or vice versa such that a free NH<sub>2</sub> can be maintained;
- (e) Ile for Leu or for Val or vice versa as roughly equivalent hydrophobic amino  
30 acids; and

hydroxylated and therefore results in an increase in the capacity of the pool of HIF to give its transactivation effect under normoxic or hypoxic conditions.

As indicated above the alteration might for a HIF protein be either in the asparagine hydroxylase binding motif or in the asparagine hydroxylase hydroxylation motif. For simplicity reference will now be made to amino acid sequence of what is thought to be approximately the total extent of the CAD domain of the two known HIF proteins HIF-1 $\alpha$  and HIF-2 $\alpha$ .

10 humanHIF-1 $\alpha$  776-SDLACRLLGQSMDESGLPQLTSYDCEVNAPIQGSRNLLQGEELLRALDQVN826  
 [SED ID NO. 2]  
 mouseHIF-2 $\alpha$  824-SGVASRLLGPSFEPYLLPELTRYDCEVNVVPVPGSSTLLQGRDLLRALDQAT-  
 874  
 [SED ID NO. 3]

15 Binding motif hydroxylation motif

It has been shown by the present inventors that under normal oxygen levels the asparagine at residue 803 of humanHIF-1 $\alpha$  and the asparagine residue at 851 of mouseHIF-2 $\alpha$  are hydroxylated in which case the transactivational domain has very low activity. If either of these two asparagines is conservatively substituted, for example, for an alanine then the CAD domain still exhibits its transactivational properties but the target asparagine is not hydroxylated. It can be seen however that adjacent the target asparagine residue of the CAD domain are very conserved amino acid sequences, these are underlined above and designated as hydroxylation motif indicating that this sequence could be considered to be an amino acid motif that is required for the asparagine hydroxylase to catalyse the hydroxylation of the target asparagine. It is postulated that substitution of these amino acids will also interfere with the hydroxylation of the target asparagine *in vivo*, and that should some or all of these be substituted conservatively then the transactivational activity of the CAD domain may be kept intact. Within this motif, the valine (V) immediately preceding the target asparagine seems particularly important, as altering this valine to alanine results in very low levels of hydroxylation in an *in vitro* hydroxylation assay (Figure 7). Alteration of other amino acids close to the asparagine have varying effects on the ability of the FIH-1 asparagine hydroxylase to modify the

hydroxylase to the CAD domain. Constitutive activity is found with such deletion in chimeric constructs (Ratcliffe *et al.*, 1998). It is anticipated that a similar finding is likely for HIF-2 $\alpha$  with constitutive expression occurring up to about amino acid 841.

5 This invention may additionally contemplate the use of an altered HIF CAD domain in a context other than for HIF functions. Specifically contemplated are alterations in the CAD domain in particular in the asparagine hydroxylation motif or other amino acids proximal the C terminus in relation to the asparagine hydroxylase binding motif. Such altered HIF CAD domain which may also have further alterations of amino acids that do  
10 not impact on the capacity of the HIF CAD domain to act as a substrate for the asparagine hydroxylase or to act as a transactivator, as contemplated above (as applicable) for the HIF protein such as for example by conservative amino acid substitutions. The second aspect contemplates chimeric proteins whereby the HIF CAD domain is adjacent a DNA binding domain other than that of HIF. This might therefore be useful in providing for a  
15 novel transcriptional enhancing molecule. Such binding proteins might be any that fall into the common classes of DNA binding domains such as basic Helix-Loop-Helix, Zinc Finger, Homeodomain, Helix-Turn-Helix.

These proteins with altered HIF CAD properties might be administered to a patient and  
20 perhaps a particular site in the patient requiring better adaptation to a hypoxic micro environment.

Another aspect of the present invention encompasses nucleic acids encoding any of the altered protein or chimeras. Typically the nucleic acid will take the form of DNA,  
25 however the nucleic acid might take the form of RNA. The nucleic acid may be expressed in a vector which may be suitable for therapeutic application. The vectors may be any one of the many available simply for replication and production of the altered protein *in vitro*. However the vector might be chosen from the type intended to introduce the nucleic acid into a patient. Possible vectors are naked DNA containing broad  
30 spectrum mammalian promoters such as  $\beta$  actin or elongation factor, or common gene

The asparagine hydroxylase of the present invention will act on HIF-1  $\alpha$  and 2 $\alpha$  in normoxic conditions and may act on other proteins with similar binding and hydroxylation motifs to those described above. The present invention also provides for a method of isolating asparagine hydroxylase enzymes, by reason of its affinity for the

5 CAD domain or portions thereof. The method may contemplate the immobilisation of the CAD domain or portion thereof, contacting the immobilised CAD domain or portion thereof with a cell extract, washing off the cell extract under mild condition, followed by washing off with more stringent conditions. The cell extract may be fractionated before

10 fractionation techniques known in protein purification protocols, such as ammonium sulphate fractionation or size fractionation by chromatographic methods. The use of a fractionated cell extract may assist with the purification. An alternative method is to immobilise a cell extract or fractionated or partially purified cell extract, by for example electrophoresing on a gel or other separation method, and then applying labelled CAD

15 domain or preferably a portion thereof to identify the concentrated band. Such bands can be cut from the gel and purified. Intracellular screens to discover a cDNA that encodes the interacting hydroxylase would include yeast or mammalian two hybrid assays.

Another general approach is to undertake a bioinformatic analysis to derive likely genes

20 that encode the enzyme. This is undertaken by the use of suitable databases of protein to look for enzymes that show sequences common to for example hydroxylases, and more specifically asparagine hydroxylases. The candidates are either cloned or obtained and purified or semi purified protein is tested by *in vitro* hydroxylation assays, binding assays or reporter gene assays in cells in methods similar to those that provided the data shown

25 in the figures 7, 8 and 3.

In the above it may be desired to use the affinity of an entire HIF CAD domain, however certain regions are not required for the asparagine hydroxylase binding and may have affinity for other matter in the cell extract or fractionated cell extract, leading to

30 difficulties with the purification. Accordingly cut down versions of the HIF CAD that

The HIF CAD domain or portion thereof may be any form to which the asparagine hydroxylase can bind and various suitable forms are discussed above, and are where the target asparagine is in an unhydroxylated state. Preferable the HIF CAD domain or portion thereof is immobilised, for example in a multiwell tray. The preparation

5 containing the asparagine hydroxylase might simply be a crude cell extract, including or excluding cell wall or cell membrane, or the preparation may be fractionated crude cell extract, various methods of fractionating the cell extract may be used and an appropriate fraction identified as having the majority of activity can be used in the preparation.

Preparations of crude or purified recombinant asparagine hydroxylases may also be used.

10 The candidate agonist or antagonist might be any molecule that might potentially be used for therapeutic purposes, including polysaccharides, peptides, nucleic acids, or other organic compounds a variety of which may be manufactured by known combinatorial approaches to screening. The preparation containing asparagine hydroxylase and the candidate agonist or antagonist are added to the immobilised HIF CAD domain or portion  
15 thereof, after a suitable incubation time, the non-immobilised material is washed off, and the HIF CAD domain or portion thereof is probed for hydroxylation of the target asparagine. The probing might be by mass spectrometry of a peptidic fragment of the HIF CAD domain or portion thereof, alternatively carbon dioxide capture assay that measures aspartyl-b-hydroxylase activity may be employed (Analytical Biochem.,  
20 vol.271, pp137-142 (1999))

The present invention might also encompass purified HIF-1 $\alpha$  and/or HIF-2 $\alpha$  or fragments thereof having the target asparagine in an unhydroxylated form.

## 25 METHODS

### *Reporter Gene Assays*

Human embryonic kidney 293T and rat pheochromocytoma PC12 cells were maintained in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% foetal calf

30 serum. For transient transfections, cells were plated onto 24 well plates at a density of

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were washed once with PBS, then lysed with binding buffer (100 mM Na-Phosphate pH8.0, 8M Urea, 0.1%NP40, 0.15M NaCl<sub>2</sub>, 5 mM imidazole, 1 mM  $\beta$ Me, 0.05 mM Na<sub>3</sub>VO<sub>4</sub>, 0.1 mM NaF, 0.5mM  $\beta$ -Glycerophosphate, protease inhibitors). Clarified lysate was then loaded onto a column containing Ni-IDA agarose (Scientifix, Australia) equilibrated in binding buffer. After extensive washing with wash buffer (100 mM Na-Phosphate pH8.0, 8M Urea, 0.5M NaCl<sub>2</sub>, 20 mM imidazole), bound proteins were eluted from the column with Elution buffer (100 mM Na- Phosphate pH8.0, 8M Urea, 200 mM imidazole). Protein sample eluted from the Ni column was then loaded onto a Butyl C4 HPLC column (Brownlee, PerkinElmer) that had been equilibrated in 0.1 % trifluoroacetic acid (TFA). HIF-2 $\alpha$ 774-874 was then subsequently eluted with increasing gradient of 80% acetonitrile, 0.1% TFA. Purified HIF-2 $\alpha$ 774-874 was then subjected to MS analysis.

An Anaerobic Workstation MkIII (Don Whitley Scientific, UK) was used to prepare hypoxic HIF- 2 $\alpha$ 774-874 protein for MS analysis. For hypoxic preparation, cells were washed and lysed inside the anaerobic workstation with buffers that had been deoxygenated overnight. After cell lysis the purification was carried out at ambient O<sub>2</sub> conditions.

20

#### *Methods for analysis of post-translational modifications*

##### *Preparation of Samples for Mass Spectrometry*

HPLC fractions, consisting of aqueous acetonitrile with 0.1% (v/v) trifluoroacetic acid, were reduced in volume to 50-100  $\mu$ l and 10  $\mu$ l of 1M NH<sub>4</sub>HCO<sub>3</sub> were added to raise the pH to approximately 8. Dithiothreitol was added to a final concentration of 2mM and the solutions were incubated for 20 min at 37 °C prior to adding iodoacetamide to a final concentration of 20mM and allowing alkylation to proceed for a further 30 min at 37 °C in the dark. Trypsin digestion was achieved by adding 1  $\mu$ g of sequencing grade modified trypsin (Boehringer) directly to the unfractionated reduction and alkylation mixture and incubation for 4 h at 37 °C. Tryptic digests were subsequently acidified by adding 50  $\mu$ l of 1% (v/v) formic acid and the contents aspirated through C18 ZipTips

*Tandem mass spectrometry (MS/MS)*

Peptides were subjected to partial sequence analysis by tandem mass spectrometry (MS/MS) in the positive ion mode using a Sciex QSTAR-Pulsar Quadrupole-quadrupole (Qq)- TOF-MS under the control of Analyst QS software. Tryptic digests were sprayed from 60% (v/v) aqueous methanol containing 0.1% (v/v) formic acid. Approximately 2 µl of diluted digests were loaded into drawn capillaries coated with gold /palladium (Protana NanoES capillaries) and fitted onto a Protana NanoES electrospray ion source. Ions were sprayed with a potential of 850 V on the sample capillary.

- 10 Collisionally-activated decomposition of peptides was achieved by selecting doubly charged ions of interest using Q1 at low resolution and manually varying the collision energy to achieve optimal spread of fragments across the desired TOF mass ranges and using nitrogen as the collision gas.
- 15 Identification of proteins was achieved by using MS/MS spectra to search the Mascot database (<http://www.matrixscience.com>) with mass error constraints of 0.1 Da and 0.05 Da for parent ions and fragments ions, respectively.

*Expression of the asparagine hydroxylase FIH-1*

- 20 FIH-1 was expressed in bacteria as a fusion protein with the maltose binding protein for rapid purification. Bacteria (BL21-CodonPlus-RIL E-coli strain from Stratagene) containing a standard IPTG inducible MBP/FIH-1 expression vector were grown at 37 °C to A600=0.8 then induced with 200µM IPTG and shaken for 6 hours 30 °C. Soluble protein was purified using amylose-agarose resin and eluting with 10 mM maltose
- 25 according to standard procedures.

*Pulldown Assays*

- The C-terminal 100 amino acids of mouse HIF-2α (ie 775-874), either wild type or the mutants N851A, or RLL to AAA, were in vitro translated in the presence of <sup>35</sup>S-
- 30 methionine from pET32 vectors using the Promega TNT translation system as

## RESULTS

### *Hydroxylation of an Asparagine residue in the C-terminus of Hypoxia Inducible Factors has a Critical Influence on Protein Function*

The Hypoxia Inducible Factor HIF-1 $\alpha$  is a ubiquitous bHLH/PAS (basic

- 5 Helix-Loop-Helix/ Per Arnt-Sim homology) transcription factor which plays a key role during adaption to low oxygen stress. Target genes for HIF-1 $\alpha$  include those of erythropoietin (EPO), vascular endothelial growth factor (VEGF) and a host of genes encoding glycolytic enzymes involved in anaerobic energy production (Semenza, 2000). Disruption of the HIF-1 $\alpha$  gene in mice has established that it is essential for
- 10 vascularisation of the embryo (Iyer *et al.*, 1997, Ryan *et al.*, 1998), while a host of studies implicate roles for HIF-1 $\alpha$  in tumour angiogenesis and the pathophysiology of ischemic disease (Semenza, 2000). A second, highly related hypoxia inducible factor, variously termed endothelial PAS protein (EPAS Semenza, 2000, Tian *et al.*, 1997), HIF-Like-Factor (HLF, Ema *et al.*, 1997) or HIF-2 $\alpha$ , is also essential during mouse
- 15 development (Tian *et al.*, 1998; Peng *et al.*, 2000), although its exact function is not understood. Two separate domains within these proteins are known acceptors of hypoxia signalling pathways. The first is the oxygen dependent degradation domain (ODD) which, at normoxia, is subject to posttranslational modification by an oxygen, Fe(II) and 2-oxoglutarate dependent prolyl hydroxylase (Ivan *et al.*, 2001; Jaakkola *et al.*, 2001).
- 20 The hydroxylated proline confers interaction with the von Hippel-Lindau ubiquitin ligase complex, resulting in ubiquitination and rapid proteolysis of the HIF proteins by the proteasome. During hypoxia, the prolyl hydroxylase ceases to function and the proteins escape surveillance of the ubiquitin-proteasome pathway, resulting in a dramatic increase of their half lives. A second region which senses hypoxia lies within the C-terminal 100
- 25 amino acids of HIF-1 $\alpha$  and HIF-2 $\alpha$ , which functions as hypoxia inducible transactivation domain (termed the CAD, C-terminal Activation Domain, Jiang *et al.*, 1997; O'Rourke *et al.*, 1999; Ema *et al.*, 1999; Carrero *et al.*, 2000; Gu *et al.*, 2001). The final C-terminal 50 amino acids of HIF-1 $\alpha$  and HIF-2 $\alpha$  show strong sequence homology (60% identity), while the preceding 50 amino acids are divergent. Chimeric proteins containing the
- 30 CADs fused to the Gal4 DNA binding domain show the CADs are silent during

enzymes. If an asparaginyl hydroxylase of this type is responsible for silencing the CADs during normoxia, its activity in cells should be blocked by dimethyl-oxalylglycine (DMOG), a cell permeable analog of 2-oxoglutarate that functions as a competitive inhibitor for this class of enzymes (Jaakkola *et al.*, 2001). Treatment of cells with DMOG resulted in activation of the Gal4DBD/HIF-1 $\alpha$  and Gal4DBD/HIF-2 $\alpha$  CAD chimeras to a similar extent as that seen during treatment with hypoxia (Figure. 6B). Moreover, mutation of the critical Asn residues to alanine, ie N803A in HIF-1 $\alpha$  and N851A in HIF-2 $\alpha$ , provided the Gal4DBD chimeras with full transcriptional activities. These activities were not increased by treatment with hypoxia, DP or DMOG (Figure 6B) and expression levels of wild type and mutant chimeras are identical during the transient transfection process (Figure 6C). The point mutants provide strong evidence that hydroxylation of the critical asparagines mediate silencing of the HIF-1 $\alpha$  and HIF-2 $\alpha$  transactivation domains. In contrast, mutation of the conserved proline within this region to alanine, ie P805A in HIF-1 $\alpha$  and P853A in HIF-2 $\alpha$ , resulted in complete loss of activities of the HIF-1 $\alpha$  and HIF-2 $\alpha$  CADs during both normoxia and hypoxia (data not shown).

These results reveal that two related but distinct processes underpin the oxygen sensing mechanism for the hypoxia inducible factors. During normoxia, oxygen dependent prolyl and asparaginyl hydroxylases modify critical residues in the ODD and CAD regions respectively, inhibiting function by conferring lability and transcriptional silencing to the proteins. When the oxygen concentration becomes limiting, activities of these enzymes is attenuated to allow increases in both HIF protein levels and intrinsic transcriptional potency. Importantly, overexpression of the HIF-1 $\alpha$  CAD in transgenic mice has been shown to attenuate tumour development, presumably by interrupting function of the endogenous HIF-1 $\alpha$  CAD (Kung *et al.*, 2000).

Prolyl hydroxylases that modify the ODD have recently been found. (Bruik and McKnight, 2001) These are novel prolyl hydroxylases that differ from the collagen prolyl hydroxylases described thus far that are located in the lumen of the ER. Likewise, the

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- Tian *et al.*, (1998) *Genes Dev.* **12**, 3320
- Zhang, J.H *et al.*, (1999) *Anal. Biochem.* **271**, 137
- Zhu and Bunn (2001) *Science*, **292**, 449-51

5. The method of screening for agonists or antagonists of the asparagine hydroxylase as in either claim 3 or 4 wherein the indicator gene is Gal 4.

6. The method of screening for agonists or antagonists of the asparagine hydroxylase as in claim 1 wherein the protein or peptide includes the asparagine hydroxylase binding motif and the asparagine hydroxylase hydroxylation motif or HIF 1 $\alpha$  or HIF 2 $\alpha$  or functional variants thereof, the inhibition or enhancement of hydroxylation of the target asparagine being measured directly by analysis of the target asparagine.

7. The method of screening for agonists or antagonists of the asparagine hydroxylase as in claim 6 wherein the analysis is by mass spectrometry.

8. The method of screening for agonists or antagonists of the asparagine hydroxylase as in claim 6 wherein the analysis is by a carbon dioxide capture assay.

9. The method of screening for agonists or antagonists of the asparagine hydroxylase as in claim 6 wherein the protein or peptide consists of a portion of a HIF protein or functional variant thereof, the portion of HIF protein including the hydroxylase binding motif and the hydroxylation motif but not having transactivational activity.

10. The method of screening for agonists or antagonists of the asparagine hydroxylase as in claim 6 wherein the portion of the HIF protein consists of the hydroxylase binding motif and the hydroxylation motif spaced apart by nine amino acids and is capable of hydroxylation by the asparagine hydroxylase.

11. The method of screening for agonists or antagonists of the asparagine hydroxylase as in claim 1 wherein the protein or peptide includes the asparagine hydroxylase binding motif and the measurement is the extent of enhancement or inhibition of binding of the asparagine hydroxylase to the binding motif.

19. A method of screening for agonists or antagonists to an asparagine hydroxylase capable of hydroxylating asparagine 851 of mouse HIF-2 $\alpha$  and asparagine 803 of human HIF-1 $\alpha$ ,

the method including of the steps of

5 contacting a peptide or protein capable of binding or being hydroxylated by the asparagine hydroxylase or the asparagine hydroxylase with a candidate agonist or antagonist, and

measuring the binding of the peptide or protein to the asparagine hydroxylase, or the peptide or protein.

10

20. The method of confirming that a candidate therapeutic molecule is an agonists or antagonists of an asparagine hydroxylase capable of hydroxylating asparagine 851 of mouse HIF-2 $\alpha$  and asparagine 803 of human HIF-1 $\alpha$ ,

the method including the steps of

15 contacting a protein or peptide including the asparagine hydroxylase binding motif and the asparagine hydroxylase hydroxylation motif or HIF 1 $\alpha$  or HIF 2 $\alpha$  or functional variants thereof, with the asparagine hydroxylase

additionally contacting the mixture with a candidate therapeutic molecule, and

20 measuring inhibition or enhancement of hydroxylation of the target asparagine directly by analysis of the target asparagine.

21. The method of screening for agonists or antagonists of the asparagine hydroxylase as in claim 20 wherein the analysis is by mass spectrometry.

25 22. The method of screening for agonists or antagonists of the asparagine hydroxylase as in claim 20 wherein the analysis is by a carbon dioxide capture assay.

23. An altered protein including a portion of the HIF CAD domain or variant thereof having a reduced susceptibility to hydroxylation of the target asparagine by an asparagine

30. The altered protein including a portion of the HIF CAD domain or variant thereof having a reduced susceptibility of hydroxylation as in claim 23 wherein additionally the binding motif is altered.

5

31 The altered protein including a portion of the HIF CAD domain or variant thereof having a reduced susceptibility of hydroxylation as in claim 23 wherein said binding motif is selected from the group consisting of [SEQ ID NO. 6] or [SEQ ID NO. 7] or an amino acid sequence of an homologous HIF 1 $\alpha$  or 2 $\alpha$  molecule.

10

32 The altered protein including a portion of the HIF CAD domain or variant thereof having a reduced susceptibility of hydroxylation as in claim 31 wherein there is a substitution of any one or more of the amino acids RLL at positions 781 to 783 of human HIF 1 $\alpha$  or the amino acids RLL at position 829 to 831 of mouse HIF 2 $\alpha$  or analogous

15 amino acids of other HIF 1 $\alpha$  or 2 $\alpha$  molecules.

33. The altered protein including a portion of the HIF CAD domain or variant thereof having a reduced susceptibility of hydroxylation as in claim 24 wherein the protein is a chimeric protein.

20

34. The altered protein including a portion of the HIF CAD domain or variant thereof having a reduced susceptibility of hydroxylation as in claim 33 where chimeric protein has a non HIF DNA binding domain.

25 35. A nucleic acid molecule with a nucleic sequence encoding the protein of claim 23.

36. A recombinant cell carrying a nucleic acid molecule of claim 35.

37. A method of screening for an asparagine dehydroxylase by binding a hydroxylated  
30 HIF  $\alpha$  and testing for CAD activity or dehydroxylation of asparagine 851 of mouse HIF

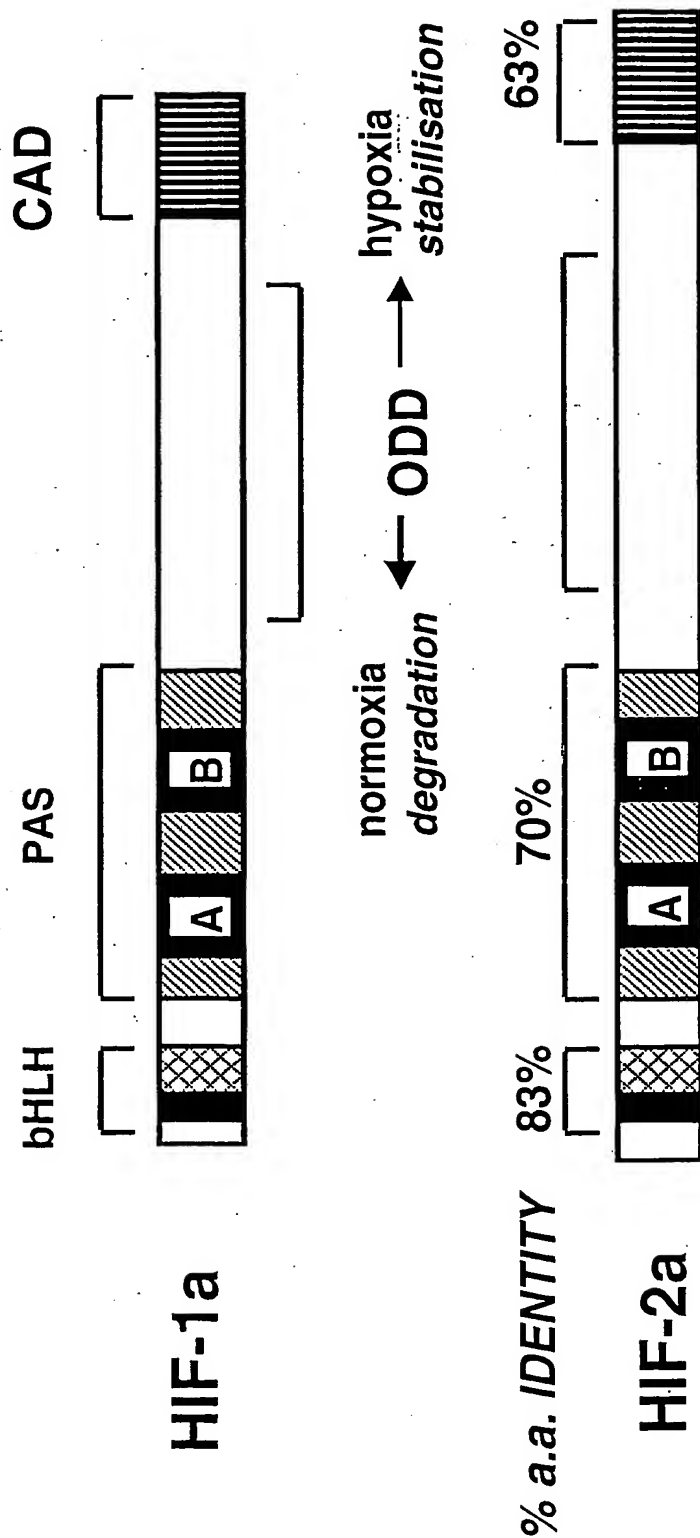


Figure 1

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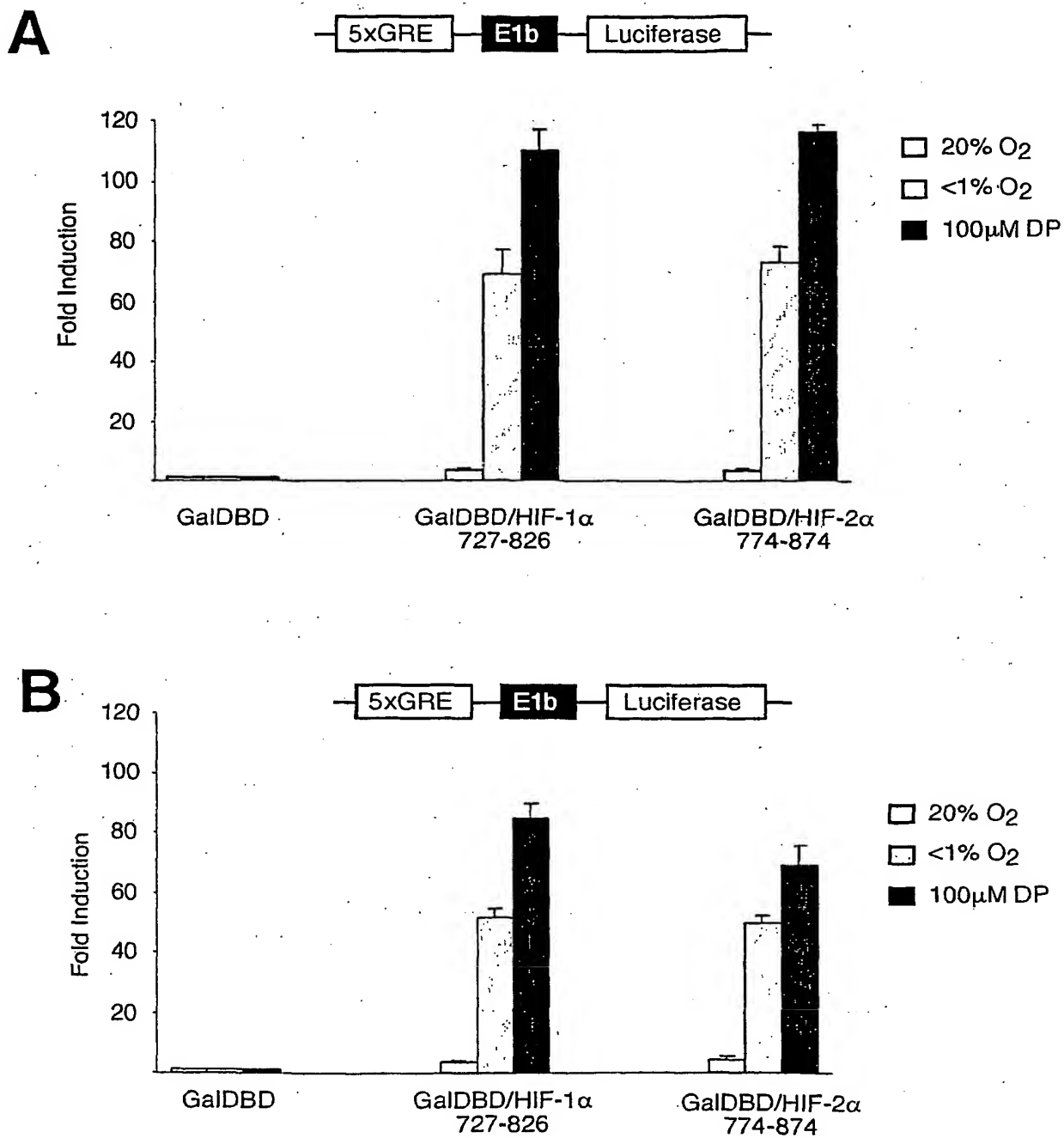


Figure 3

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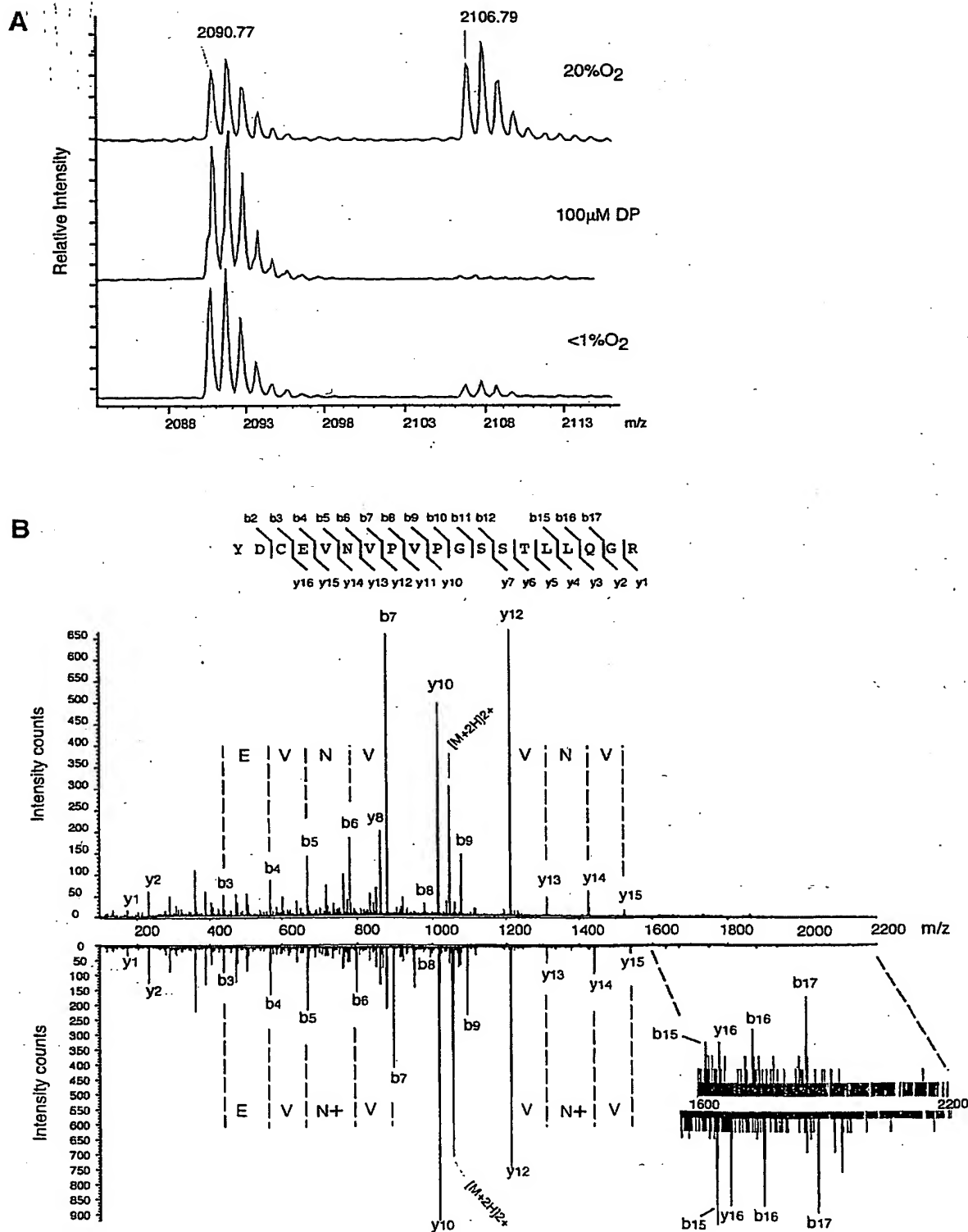
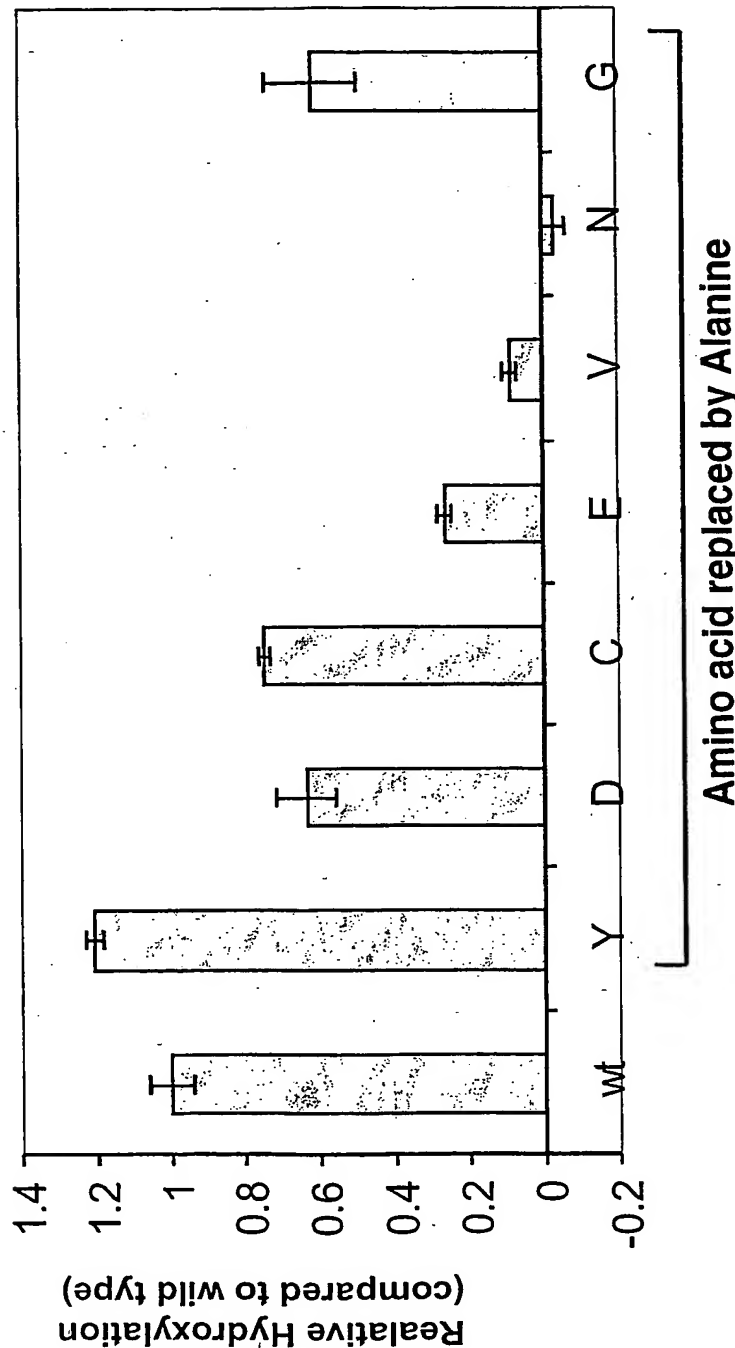


Figure 5

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# Importance of individual amino acids within the HIF CAD asparagine hydroxylation motif



YDCEVNxxxxG - Amino acids within the CAD  
hydroxylation motif selected for alteration

Figure 7

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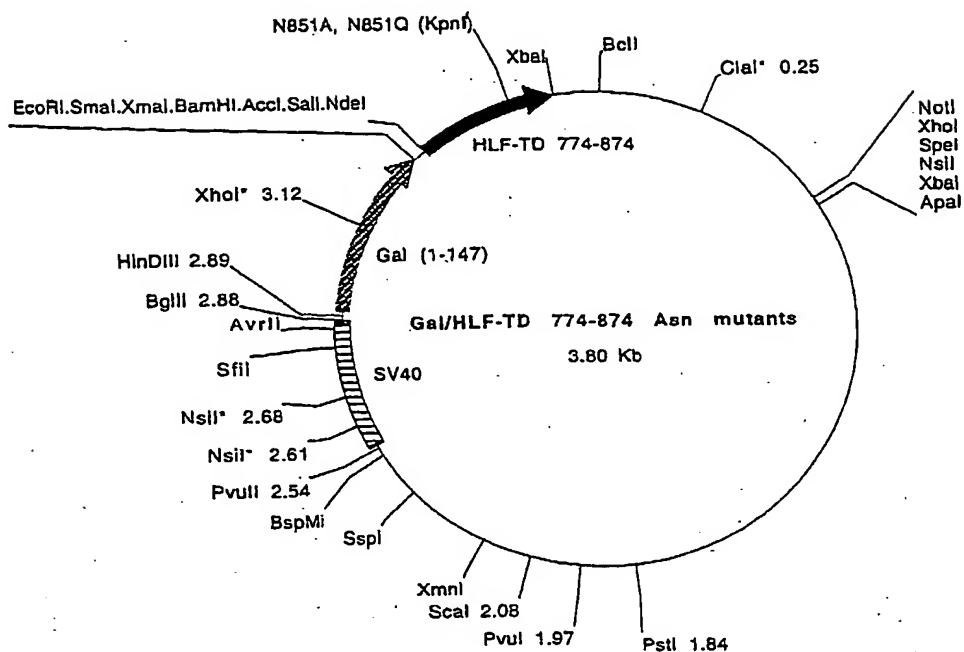


FIGURE 9A

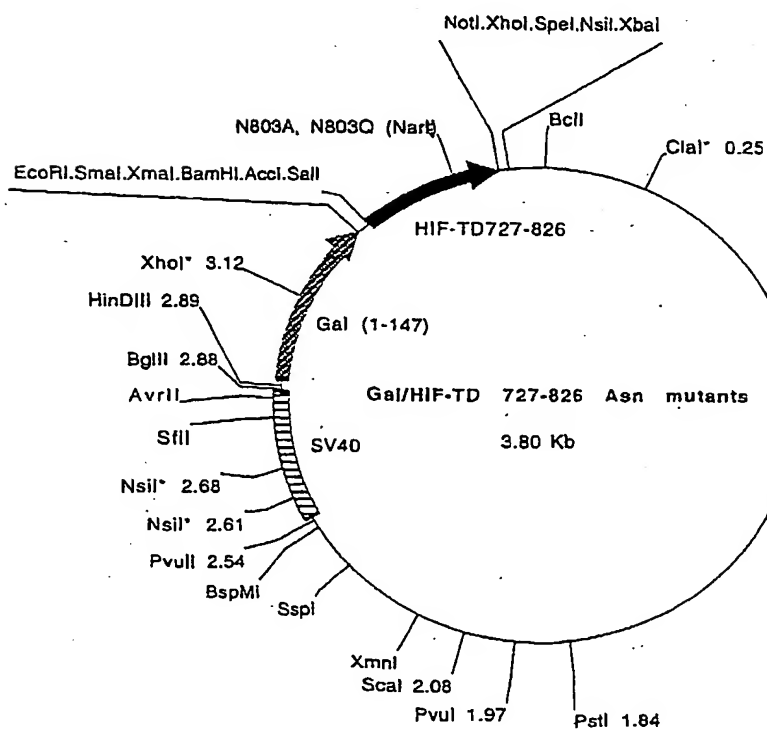


FIGURE 9B

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## Sequence Listing

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 <211> 20  
 <212> PRT  
 <213> Mus musculus  
 10 <220> coding sequence  
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 1 5 10 15  
 15 Leu Leu Gln Gly Arg  
 20  
 <210> 2  
 <211> 51  
 <212> PRT  
 20 <213> Human  
 <223> portion of HIF 1 alpha gene including asparagine that is hydroxylated  
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 1 5 10 15  
 25 Gly Leu Pro Gln Leu Thr Ser Tyr Asp Cys Glu Val Asn Ala Pro  
 20 25 30  
 Ile Gln Gly Ser Arg Asn Leu Leu Gln Gly Glu Glu Leu Leu Arg  
 35 40 45  
 Ala Leu Asp Gln Val Asn  
 30 50  
 <210> 3  
 <211> 51  
 <212> PRT  
 <213> Mus musculus  
 35 <223> portion of HIF 2 alpha gene including asparagine that is hydroxylated  
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 Val Pro Gly Ser Ser Thr Leu Leu Gln Gly Arg Asp Leu Leu Arg  
 35 40 45  
 Ala Leu Asp Gln Ala Thr  
 50  
 45 <210> 4  
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 Asn Leu  
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 1 5 10 15  
 Thr Leu  
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 <211> 8

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5	ctaggaggtc	tccacttcca	gtagcaatgg	gagggggaga	agaagctcgt	aaacggcttt	2580
	gaagatgaaa	caggcctgag	gccgagattg	ttgacacagc	tctactgaat	aggcaaacag	2640
	ttggctctta	agaggccagg	gtgatgcaa	gccaataaaa	tgcagctgtt	gtctctttgc	2700
	tgcccccttt	actgccagct	atcctggtgc	ataaaagggc	ctgccacagc	tcagggagca	2760
	caggcctttg	gctcagtcct	ctgccagctt	ctccactgtc	cagacacctc	cctgtcgaca	2820
10	acatg						

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/AU02/01290

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,Y	Groulx Isabelle, et.al., " <i>Oxygen-Dependent Ubiquitination and Degradation of Hypoxia-Inducible Factor Requires Nuclear-Cytoplasmic Trafficking of the von Hippel-Lindau Tumor Suppressor Protein</i> ", Molecular and Cellular Biology, (Aug 2002) <u>22</u> (15) pp.5319-36 See in particular page 5320 column 2 lines 2-5	1-22
P,Y	Dames Sonja A., et. al., " <i>Structural basis for Hif-1 <math>\alpha</math>/CBP recognition in the cellular hypoxic response</i> ", Proceedings of the National Academy of Sciences of The United States of America ( 16 Apr 2002) <u>99</u> (8) pp.5271 -6 See in particular page 5274 column 2 line 41 to page 5276	1-22
A	Jaakola P., et. al., " <i>Targeting of HIF-<math>\alpha</math> to the von Hippel-Lindau Ubiquitylation Complex by O<sub>2</sub>-Regulated Prolyl Hydroxylation</i> ", Science, (20 Apr 2001) <u>292</u> (5516) pp.468-72	